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Application No. 09/770,943
Exhibit 1

Immunostimulatory DNA-based vaccines induce cytotoxic lymphocyte activity by a T-helper cell-independent mechanism

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Received 15 October 1999; accepted 8 March 2000

Immunostimulatory DNA sequences (ISS) contain unmethylated CpG dinucleotides within a defined motif. Immunization with ISS-based vaccines has been shown to induce high antigen-specific cytotoxic lymphocyte (CTL) activity and a T_h1-biased immune response. We have developed a novel ISS-based vaccine composed of ovalbumin (OVA) chemically conjugated to ISS–oligodeoxynucleotide (ODN). Protein–ISS conjugate (PIC) is more potent in priming CTL activity and T_h1-biased immunity than other ISS-based vaccines. Cytotoxic lymphocyte activation by ISS-ODN-based vaccines is preserved in both $CD4^{-/-}$ and MHC class $II^{-/-}$ gene-deficient animals. Furthermore, PIC provides protection against a lethal burden of OVA-expressing tumor cells in a $CD8^+$ cell-dependent manner. These results demonstrate that PIC acts through two unique mechanisms: T-helper-independent activation of CTL and facilitation of exogenous antigen presentation on MHC class I. This technology may have clinical applications in cancer therapy and in stimulating host defense in AIDS and chronic immunosuppression.

Keywords: immunostimulatory DNA sequences, CpG motif, CTL activation, AIDS

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Immunostimulatory DNA sequences (ISS), or CpG motifs, contain unmethylated CpG dinucleotides in a consensus motif of 5′-pur-pur-CpG-pyr-pyr-3′ (refs 1,2). These sequences were originally isolated as the antitumor principle from *Mycobacterium* bacille Calmette-Guérin (BCG), a therapeutic agent commonly used in the treatment of bladder carcinoma[3,4]. Immunostimulatory DNA sequences exert a number of stimulatory effects upon natural killer cells[5] and antigen-presenting cells (APC) such as macrophages, dendritic cells, and B cells[6–8], including upregulation of proinflammatory cytokines interleukin 6 (IL-6), IL-12, and interferon γ (IFNγ), and cell surface molecules major histocompatibility complex (MHC) class I, class II, CD40, B7-1, B7-2, and ICAM-1. By these criteria, ISS have little direct effect upon either $CD4^+$ or $CD8^+$ T cells[6]. Despite this, immunization with ISS-based vaccines such as plasmid DNA[9,10] or protein, and ISS–oligodeoxynucleotide (ODN) coadministration[11–14], results in two T-cell phenotypes: antigen-specific cytotoxic lymphocyte (CTL) activity and a T_h1-biased immune response.

These observations raise three questions regarding the stimulatory effect of ISS. First, ISS do not directly affect T cells[6], yet T-cell phenotypes (CTL and T_h1-biased immunity) are cardinal features of vaccination with ISS adjuvants. How is the ISS stimulus transduced to the T cells? Second, are CTL activation and the T_h1 bias interdependent or not? Third, what are the relative contributions of $CD4^+$ and $CD8^+$ cells to tumor immunity?

These questions directly address the concept of "cross-priming," the phenomenon of priming CTL against foreign protein antigens. It has been shown that $CD4^+$ T-helper activity is required to prime CTL against specific antigens on allogeneic tumor cells in a mouse model of tissue rejection[15]. A three-cell interaction between APC, T helper, and CTL was required to generate the necessary lytic activity[16]. Current models of cross-priming posit that APC must have an initial "licensing" interaction with T-helper cells before they can activate CTL[17–19]. The licensing step requires CD40–CD40 ligand interaction between APC and T-helper cells[17,19]. The licensing and activation steps may be dissociated both spatially and temporally, with the APC acting as a "conditioned bridge" between the T_h and CTL[18]. However, ISS stimulation affects both T_h and CTL, suggesting that the signal is transduced through APC that interact with both populations. Immunostimulatory DNA sequence stimulation may replace some or all of the "licensing" effects on APC, in which case the T_h1 phenotype and CTL activation may be independent. Antigen-presenting cells that not only present antigen on MHC class I but have also been "licensed" by ISS stimulation should be able to directly activate antigen-specific CTL activity. This hypothesis requires that both ISS and protein antigen must be colocalized to the same APC.

Here we describe a novel ISS-based vaccine: protein–ISS conjugate (PIC) of a model antigen, ovalbumin (OVA), chemically linked to ISS–ODN. This reagent is designed to efficiently deliver ISS–ODN and antigen to the same APC. In this report, it is shown that PIC is more efficient than other ISS-based vaccines in stimulating CTL activity and T_h1-biased immunity, and that activation of CTL is independent of MHC class II-restricted T-cell help. The in vivo efficacy of PIC vaccination is demonstrated by its ability to stimulate protective, $CD4^+$-independent immunity in a murine cancer model.

Results

Synthesis of OVA–ISS conjugates. The OVA–ISS conjugates were prepared as described in the Experimental Protocol (Fig. 1A). Nonreducing SDS–PAGE was used for qualitative evaluation of PIC. Bands corresponding to PIC were visualized by short-wave UV shad-

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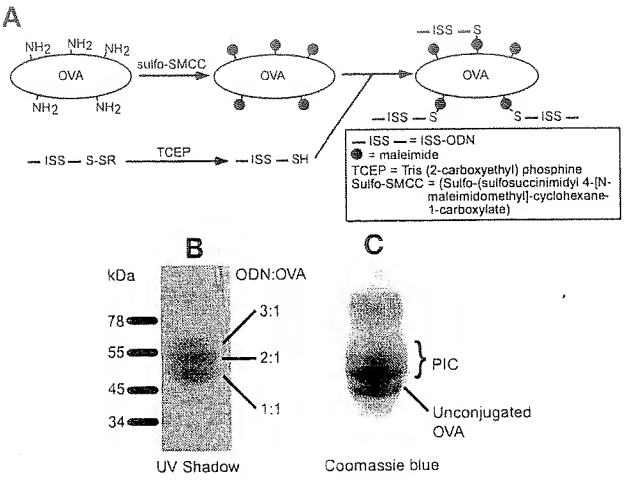

Figure 1. Synthesis of ovalbumin–ISS–ODN conjugate. (A) Schema for PIC synthesis. Disulfide-linked phosphorothioate ISS–ODN and chicken OVA were modified as described in the text and mixed at a 5:1 (ISS–ODN:OVA) molar ratio. The resulting PIC was analyzed by SDS–PAGE (10 to 20% gradient gel). (B) Short-wave UV shadowing. Bands are visible corresponding to 1:1, 2:1, and 3:1 ODN:OVA ratio conjugate. Unconjugated OVA is not visualized by this method. (C) The same gel as in (B) was stained with Coomassie G-250. A band corresponding to unconjugated OVA is visible at ~45 kDa. Bands corresponding to PIC >1:1 ratio stain poorly because of increased ODN concentration.

owing on a silica gel thin-layer chromatography plate (Fig. 1B), followed by Coomassie G-250 staining to detect protein bands (Fig. 1C). A ladder is visible corresponding to increasing ISS–ODN:OVA ratios in the conjugate (Fig. 1B). Protein–ISS conjugate at ISS–ODN:OVA ratios higher than 1:1 stains poorly with Coomassie blue (Fig. 1C) because of the high concentration of acidic ODN. The average molar ratio of ISS–ODN:OVA in this series of experiments was ~2.4:1. Conjugate was also synthesized with mutated ODN that do not contain CpG dinucleotides, and this mutated PIC (mPIC) was used as a control.

PIC vaccination induces high antigen-specific CTL activity. To determine if PIC is more efficient than coadministration of OVA and ISS–ODN or plasmid DNA vaccine, wild-type (wt) C57BL/6 mice were immunized with PIC and the resultant CTL activity was evaluated. For comparison, test groups were immunized with mPIC, OVA + ISS–ODN coadministration, pACB–OVA (a plasmid DNA vaccine that contains ISS motifs), and OVA alone. An untreated group was also included. In secondary CTL assays, PIC vaccination resulted in remarkably high activity, with 91 ± 5% specific lysis observed at a 25:1 effector-to-target (E:T) ratio and 92 ± 4% at a 1:1 ratio, indicating that activity was at a plateau even at a high dilution (Fig. 2). At a 0.2:1 E:T ratio, specific lysis was 64 ± 10%. This activity was significantly higher than that observed in OVA + ISS coadministration, despite the higher molar ratio of ISS–ODN:OVA protein in the latter treatment (6:1 in the coadministration versus ~2.4:1 in the PIC).

Protein–ISS conjugate also elicited higher levels of CTL activity than pACB–OVA vaccination. Animals immunized with mPIC exhibited low CTL activity (33 ± 2% specific lysis at 25:1 ratio), which was comparable to the nonspecific adjuvant activity of mutated oligonucleotide previously observed[12]. Ovalbumin administration alone did not stimulate CTL activity. Target cells loaded with an irrelevant MHC class I-restricted peptide were not lysed by splenocytes from OVA–PIC-immunized mice, indicating that the observed response was antigen-specific. These results show that vaccination with PIC resulted in higher antigen-specific CTL activity than other ISS-based vaccines.

PIC vaccination induces a T_h1-like immune response. Total splenocyte cytokine production and antigen-specific isotype switching to IgG_{2a} were examined to assess the T-helper response to PIC vaccination. Splenocytes from the groups described in the previous section were restimulated in culture, and tissue culture supernates were collected at day 3 for cytokine enzyme-linked immunosorbent assay (ELISA). Both PIC and OVA + ISS–ODN coadministration induced comparable levels (3,800 ± 1,800 and 3,600 ± 1,100 pg ml^{-1}, respectively) of IFNγ, a T_h1-associated cytokine, in response to OVA stimulation (Fig. 3A). By comparison, pACB–OVA and mPIC vaccination resulted in IFNγ production approximately 20% and 10% of this amount, respectively. The T_h2-associated cytokine IL-4 was not detected in any of the groups (data not shown).

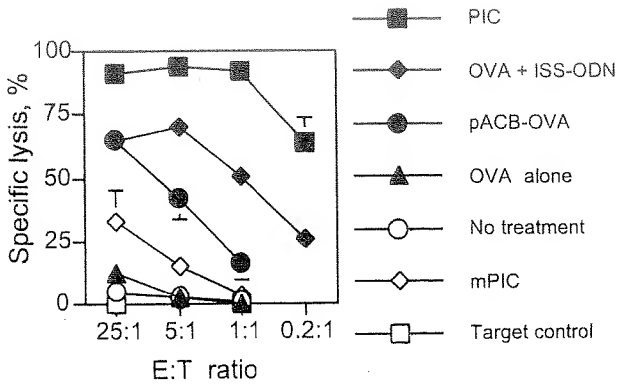

Figure 2. Antigen-specific cytotoxic lymphocyte activity following intradermal immunization of test animals on days zero and 14 with the following vaccines: PIC (50 μg, ■), OVA + ISS–ODN coadministration (50 μg each, ◆), mPIC (50 μg, ◇), pACB–OVA (50 μg, ●, or OVA alone (50 μg, ▲). Vaccines were diluted in sterile normal saline solution. Total splenocytes were isolated after six weeks and restimulated in culture for five days. Secondary CTL activity was determined by lactate dehydrogenase release. Targets were EL-4 cells loaded with either MHC class I-restricted OVA peptide or influenza virus nucleoprotein peptide (target control, □). Error bars indicate s.e.m. Data are averaged from four to five mice per group, and are representative of four experiments.

Interferon γ is a switch factor for IgG_{2a} (ref. 20), and isotype switching to IgG_{2a} is also a marker for T_h1-biased immune responses. Serum was collected at week 6 and assayed for OVA-specific IgG_1 and IgG_{2a} by immunoglobulin ELISA. Protein–ISS conjugate induced a substantial isotype switch to IgG_{2a} (Fig. 3B, C), similar to coadministration of OVA + ISS–ODN. Vaccination with PIC also produced a higher titer of IgG_1 + IgG_{2a}, suggesting that the magnitude of the T_h- and concomitant B-cell response was higher. Immunization with mPIC resulted in antigen-specific IgG_1 production without isotype switching to IgG_{2a}. These data indicate that PIC vaccination promot-

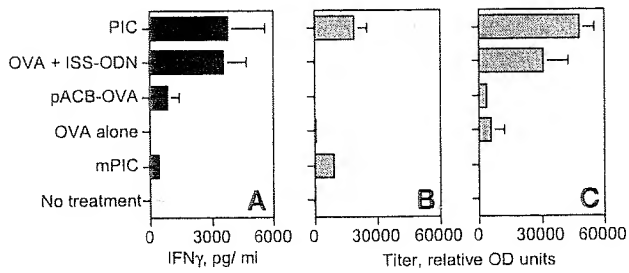

Figure 3. PIC vaccination induces a T_h1-biased immune response. (A) IFNγ production in total splenocyte restimulation assay. Total splenocytes were isolated as described in Figure 2. Splenocytes were restimulated as described in the text, and IFNγ concentration in day 3 supernates was determined by cytokine ELISA. (B) IgG_1 and (C) IgG_{2a} titers in week 6 serum from immunized mice. Relative titer was determined by isotype-specific ELISA. Error bars indicate s.e.m. Data are averaged from four to five mice per group, and are representative of four experiments.

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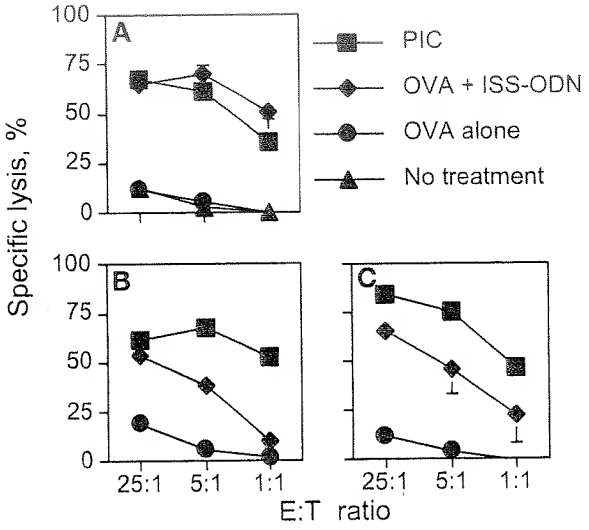

Figure 4. Activation of CTL activity by PIC is independent of MHC class II-restricted help. (A) Wild type (B) $CD4^{-/-}$, and (C) MHC class $II^{-/-}$ gene-deficient animals were vaccinated intradermally at the tail base on days zero and 14 with either PIC (50 μg, ■), OVA + ISS–ODN (50 μg each, ◆, or OVA alone (50 μg, ●). Cytotoxic lymphocyte activity was determined as described in Figure 2. Error bars indicate s.e.m. Data are averaged from four mice per group, and are representative of two experiments.

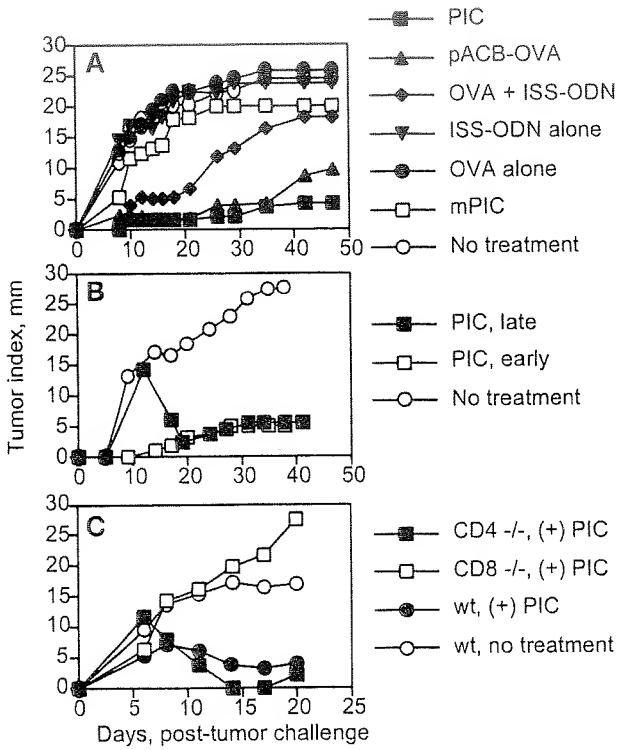

Figure 5. Plot showing that PIC vaccination provides protective immunity in both preventive and therapeutic models of cancer. (A) Preventive model. Test animals were vaccinated intradermally at the tail base on days zero and 14 with the following vaccines: PIC (50 μg, ■), mPIC (50 μg, □), OVA + ISS–ODN coadministration (50 μg each, ◆), pACB–OVA (50 μg, ▲), or OVA alone (50 μg, ●). On day 28, each group received a lethal challenge of 20×10^6 E.G7–OVA cells subcutaneously in the right flank, and tumor growth was followed over the subsequent six weeks. The observed difference in tumor growth is statistically significant between the PIC and mPIC groups (two-tailed t test, $p = 0.05$), PIC and OVA alone ($p < 0.02$), and PIC and no treatment ($p < 0.025$). (B) Therapeutic model. Test animals received subcutaneous tumor challenge on day zero, and early (days zero, 6, and 11, □) or late (days 6, 11, and 15, ■) intradermal vaccination with PIC (50 μg). The observed difference in tumor growth between the PIC treatment groups and untreated controls is statistically significant ($p < 0.005$). (C) Therapeutic model in $CD4^{-/-}$ (■) and $CD8^{-/-}$ (□) gene-deficient mice. Gene-deficient animals received subcutaneous tumor challenge on day zero and were immunized intradermally with PIC (50 μg) on days zero, 3, and 7. The observed difference in tumor growth between $CD4^{-/-}$ and $CD8^{-/-}$ groups is statistically significant ($p < 0.005$). Tumor growth in all three plots is expressed as tumor index = square root (length × width). Data are averaged from six mice per group and are representative of two experiments each.

ed a T_h1-like helper phenotype as measured by IFNγ production and IgG_{2a} isotype switching.

Induction of CTL activity by PIC is independent of MHC class II-restricted help. To test the hypothesis that CTL induction and T_h1 bias are independent in PIC vaccination, $CD4^{-/-}$ and MHC class $II^{-/-}$ gene-deficient mice were vaccinated according to the protocol described earlier. We included MHC class $II^{-/-}$ mice to assess the contribution of MHC class II-restricted T-cell help by $CD4^+/CD8^+$ lymphocytes. The CTL responses of wt mice to PIC, OVA and ISS–ODN coadministration, and OVA alone are shown in Figure 4A. Vaccination with either PIC or OVA + ISS–ODN elicited high antigen-specific CTL activity (61 ± 3% and 54 ± 2% specific lysis at a 25:1 E:T ratio, respectively) from $CD4^{-/-}$ mice (Fig. 4B). These vaccines also stimulated CTL activity (83 ± 3% and 65 ± 4% specific lysis, respectively) from MHC class $II^{-/-}$ mice (Fig. 4C). Ovalbumin alone did not elicit CTL activity in these groups. Plasmid pACB–OVA did not stimulate antigen-specific CTL activity in $CD4^{-/-}$ mice (data not shown). As expected, neither $CD4^{-/-}$ nor MHC class $II^{-/-}$ mice generated a T_h1-biased immune response to PIC vaccination as measured by IFNγ and IgG_{2a} production (data not shown). Therefore, activation of CTL activity by ISS adjuvant was independent of MHC class II-restricted T-cell help. Interestingly, effector function from $CD4^{-/-}$ and MHC class $II^{-/-}$ mice immunized with OVA + ISS–ODN was more rapid diluted at 5:1 and 1:1 E:T ratios compared to PIC vaccination, suggesting that coadministration was less efficient than PIC under conditions where T-cell help was not available.

Vaccination with PIC results in protective immunity in mouse models of cancer. To assess the in vivo effectiveness of PIC vaccination, two mouse models of cancer were examined. In a preventive model of tumor vaccination, C57BL/6 mice were vaccinated with PIC and other ISS-based vaccines, as well as controls. The test animals were vaccinated twice, then received a lethal tumor challenge of E.G7–OVA or EL-4 cells[21], and tumor growth was followed for six weeks.

Vaccination with PIC suppressed tumor growth (Fig. 5A). Vaccination with pACB–OVA also inhibited tumor growth, but to a lesser degree. Although OVA + ISS–ODN initially appeared to slow tumor growth, at later time points this effect was reduced. Neither ISS–ODN nor OVA protein alone appeared to significantly retard tumor growth. Similarly, vaccination with mPIC, which does not contain CpG dinucleotides, did not confer protection. Immunization with ISS-based vaccines did not prevent the growth of EL-4 cells, the parental line that does not express OVA, indicating that the protective effect was antigen-specific (data not shown). Treatment with ISS-based vaccines also did not appear to affect overall survival, in that there was no evidence of increased, non-tumor-related morbidity or mortality among groups that demonstrated tumor immunity when compared to test groups that were susceptible to tumor growth.

Because PIC appeared to be the most effective vaccine for stimulating resistance to tumor growth in the preventive model, it was also tested in a therapeutic model of cancer. Test animals received

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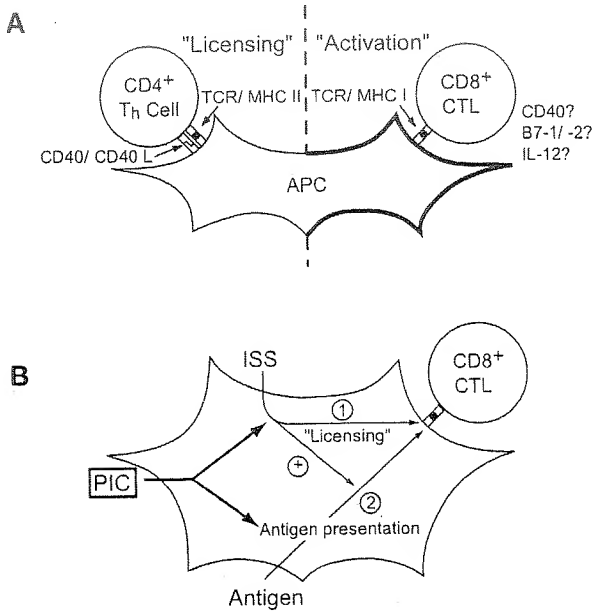

Figure 6. ISS-based vaccines provide T-helper-independent "licensing" of APC and facilitation of exogenous antigen presentation on MHC class I. (A) Licensing model of cross-priming CTL activity to soluble protein antigen. CTL activation is dependent upon an initial licensing interaction between APC and CD4+ T-helper cells. This licensing step is dependent upon CD40–CD40 ligand interaction, and confers competence for APC to activate CD8+ CTL. Figure is adapted from ref. 21. (B) Model of ISS-based vaccine action suggested by data presented in this report. (1) ISS–ODN appears to replace some or all of the licensing functions of T-helper cells in CTL activation. This may involve upregulation of APC surface expression of CD40 and/or B7-1/B7-2, as well as increased IL-12 production. (2) ISS–ODN appears to facilitate the presentation of exogenous antigen on MHC class I, possibly through TAP-dependent endosome-to-cytosol shuttling. This is unlike the common presentation of exogenous antigens on MHC class II. The reason that PIC is more efficient than OVA + ISS–ODN coadministration in the absence of T-cell help is that colocalization of ISS–ODN and antigen into the same APC is achieved in a single step.

tumor challenge on day zero, and were subsequently immunized either on days zero, 6, and 11 (early), or on days 6, 11, and 15 (late). Early vaccination with PIC resulted in profound suppression of tumor growth relative to controls (Fig. 5B). Late vaccination with PIC induced tumor regression by 14 days, with subsequent suppression of tumor growth. These results showed that vaccination with PIC resulted in protective immunity against tumor expressing OVA in both preventive and therapeutic models of cancer.

PIC-induced antitumor immunity is dependent on $CD8^+$ CTL and independent of $CD4^+$ cell help. Protein–ISS conjugate efficiently promotes CTL activity and T_h1-biased immune responses. The relative contributions of $CD8^+$ CTL activity and $CD4^+$ T_h-dependent mechanisms are unclear in this system. To assess their respective roles in antitumor immunity, $CD4^{-/-}$ and $CD8^{-/-}$ gene-deficient mice received subcutaneous tumor challenge on day zero and were immunized with PIC on days zero, 3, and 7. $CD4^{-/-}$ and wt control animals exhibited similar suppression of tumor growth, whereas $CD8^{-/-}$ mice did not suppress tumor growth (Fig. 5C). As expected, $CD4^{-/-}$ animals did not exhibit a T_h1-biased immune response, whereas $CD8^{-/-}$ mice had a response similar to wt animals (data not shown). These results showed that protective antitumor immunity induced by PIC is mediated by $CD8^+$ CTL activity, rather than T_h-dependent mechanisms.

Discussion

In this report, a novel PIC vaccine is shown to stimulate both high antigen-specific CTL activity and a T_h1-biased immune response. Activation of CTL by this vaccine is independent of MHC class II-restricted T-cell help. In addition, PIC vaccination provides protection in both preventive and therapeutic models of cancer. These results directly illuminate a central issue in the investigation of ISS, which is the role of T_h cells in the activation of CTL by ISS-based vaccines. The data presented show that the CTL and T_h responses to ISS–ODN-based vaccines are independent, and that it is the CTL response that confers protective immunity against OVA-expressing tumor in the presented models. These data also suggest that APC interaction is the common pathway through which immunostimulatory signals from ISS are transduced to the adaptive immune response.

Antitumor immunity induced by ISS-based vaccines is dependent upon $CD8^+$ CTL activity, as demonstrated by protection against tumor in $CD4^{-/-}$ animals and lack of protection in $CD8^{-/-}$ mice. However, a simple correlation between in vitro CTL activity and tumor protection is not evident. OVA + ISS coadministration also elicits high CTL activity without T_h-cell help, but only intermediate tumor protection. DNA vaccination with pACB–OVA stimulates intermediate CTL activity in wt mice and low activity in $CD4^{-/-}$ mice, but provides tumor protection similar to PIC. It is likely that the development of in vivo tumor protection involves complex interactions between host, vaccine, and tumor. Nevertheless, PIC appears to be most efficient in stimulating protective antitumor immunity.

The rapid kinetics of antitumor immunity induced by PIC is also notable. In the therapeutic model, concomitant tumor challenge and vaccination completely suppressed tumor growth, whereas treatment initiated six days after tumor challenge resulted in complete regression of tumor by day 14. This could potentially be attributed to the T_h-independent mechanism of CTL priming, but further investigation is needed to determine what other factors mediate this rapid response.

Current models of cross-priming consist of two steps: a "licensing" interaction between APC and T_h, followed by an activating interaction between "licensed" APC and CTL (Fig. 6A)[17–19,22]. The licensing step is dependent upon CD40–CD40 ligand interaction[17,19]. The requirements for the activation step are not fully elucidated, but current data suggest that CD40, B7-1, B7-2, and IL-12 signaling may all play roles[23–26]. The activation of CTL in the absence of T-cell help strongly suggests PIC can replace the "licensing" functions of the T_h–APC cell interaction (Fig. 6B, arrow 1). Current data indicate that ISS stimulation can upregulate a number of proinflammatory cytokines and surface molecules, which may account for this effect[6,27]. Investigation into the roles that these molecules play in direct activation may provide mechanistic insight into this phenomenon.

The data presented also demand that soluble exogenous antigen be presented on MHC class I in order to engage the T-cell receptor on $CD8^+$ CTL (Fig. 6B, arrow 2). It appears that ISS facilitates exogenous antigen presentation, a unique function that has not been observed in other adjuvants. Presentation of exogenous protein antigens on MHC class I has been observed in vitro, but its significance in vivo is uncertain. Three mechanisms for this phenomenon have been demonstrated: a transporter associated with antigen presentation (TAP)-dependent pathway, also known as endosome-to-cytosol shuttling[28,29], a TAP-independent, or "regurgitant," pathway[30,31], and a "recycling" pathway[32]. Though subtle, the implications of this finding are profound, because it may provide a window to delineate the regulation of exogenous antigen presentation and the role it plays in adaptive immunity.

Ovalbumin–PIC stimulates a remarkable level of CTL activity, and similar results have been demonstrated with ISS–ODN conjugated to β-galactosidase or HIVgp-120 (Tighe et al., manuscript submitted). Why is PIC more efficient than OVA + ISS–ODN

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coadministration despite a lower molar ratio of ISS–ODN:OVA? A potential mechanism is suggested by the $CD4^{-/-}$ and MHC class $II^{-/-}$ mice. These animals exhibited a reduction in CTL activation when compared to similar mice immunized with PIC (Fig. 4B, C). An intriguing interpretation of this result is that direct activation of CTL in this system requires colocalization of protein antigen and ISS in the same APC. Thus, the licensing function and exogenous antigen presentation on MHC class I are coupled to activate $CD8^+$ cells (Fig. 6B). Under suboptimal conditions such as absence of T-cell help, direct activation by PIC is more efficient than OVA + ISS–ODN separately, because colocalization is achieved in a single step.

These results suggest a promising role for ISS-based vaccines in cancer immunotherapy. However, this system used an artificial tumor-specific antigen (OVA), and the applicability of this technology to clinical use will depend upon the demonstration of efficacy with naturally occurring human tumor-associated antigens. Nevertheless, these results are encouraging, because ISS-induced antitumor immunity has characteristics that may counteract mechanisms that allow tumors to evade the immune system. For example, ISS-induced immunity is independent of T-cell help, which may counteract antiinflammatory mediators expressed by tumors that negatively affect T_h-cell function[33,34]. The very high CTL activity stimulated by PIC may overcome the downregulation of MHC class I by some tumors[35–37]. Interestingly, these results bring ISS full circle to its origin from the antitumor agent BCG[3,4]. Application of this technology to clinical use merits further investigation, since clinical efficacy in the context of bladder carcinoma has already been demonstrated.

Immunostimulatory DNA sequence-based vaccines may have another clinical application in AIDS and other immunodeficiencies, which are characterized by reduced or absent T_h function. These vaccines could induce effective cell-mediated immunity independent of T-cell help, providing protection against opportunistic infection. They may also be effective against HIV itself.

Industrial-scale synthesis of PIC vaccines would be limited by the supply and expense of producing recombinant antigen and phosphorothioate ODN. The reagents for conjugation are relatively inexpensive, and the method is simple. Quality control could be easily achieved by the methods presented in the text. However, important issues, such as safety and efficacy in humans and long-term stability of PIC vaccines, remain to be addressed.

In summary, the results presented here show that PIC vaccines can induce protective antitumor CTL immunity by two unique mechanisms. First, they replace the licensing function of T-helper cells, and second, they facilitate the presentation of exogenous antigens on MHC class I. Because of their efficacy and independence from T-cell help, ISS-based vaccines may have clinical applications in cancer and infectious disease.

Experimental protocol

Mice. C57BL/6 J and $CD4^{-/-}$ mice were purchased from Jackson Laboratories (Bar Harbor, ME). MHC class $II^{-/-}$ gene-deficient mice were purchased from Taconic Farms (Germantown, NY). $CD8^{-/-}$ gene-deficient mice were the generous gift of Wei Ping Feung-Leung and Tak Mak.

Cell lines and tissue culture. E.G7–OVA cells were the generous gift of Angel Porgador and Eli Gilboa. Cell lines were cultivated in Dulbecco's modified Eagle's medium (DMEM), high glucose (Cellgro, Natham, VA) supplemented with 10% (vol/vol) heat-inactivated fetal calf serum (FCS; Gibco BRL, Gaithersburg, MD), 2 mM L-glutamine (Cellgro), and 100 U ml^{-1} penicillin and 100 µg ml^{-1} streptomycin (Pen/Strep; Cellgro). E.G7–OVA medium also contained 400 µg ml^{-1} G418 (Calbiochem, San Diego, CA). Cells were grown in 250 ml tissue culture flasks (Falcon, Franklin Lakes, NJ) at 37°C, 5% CO_2.

Protein ISS–ODN conjugate synthesis. All chemicals are from Sigma (St. Louis, MO) unless otherwise noted. Ovalbumin (chicken egg albumin, grade VI) was activated with a 20-fold molar excess of sulfosuccinimidyl-4-(*N*-maleimidomethyl) cyclohexane-1-carboxylate (sulfo-SMCC; Pierce, Rockford, IL) at room temperature for 1 h. This modified the amino side chains of L-lysine residues by the addition of maleimide groups. Residual reagents were removed by chromatography on a G-25 desalting column (Amersham Pharmacia Biotech, Piscataway, NJ). 5′-disulfide-ISS–ODN were reduced with 200 mM Tris-(2-carboxyethyl) phosphine (TCEP; Pierce) at room temperature for 1 h, and residual reagents were removed by chromatography on a G-25 desalting column. The resulting 5′-thio-ISS–ODN were mixed with the modified OVA at a 5:1 molar ratio (ISS–ODN:OVA) and incubated overnight at room temperature. 5′-Disulfide-linked phosphorothioate ISS–ODN, sequence 5′-disulfide-TGACTGTGAACGTTCGAGATGA-3′, and mutated ODN, sequence 5′-disulfide-TGACTGTGAACCTTCGAGATGA-3′ were purchased from Tri-Link Biotechnology (San Diego, CA). Nonreducing SDS–PAGE was performed using Novex (San Diego, CA) 10–20% Tricine minigels run at a constant voltage of 100 V. Protein concentration was determined by Bradford assay (Bio-Rad, Hercules, CA). Protein–ISS conjugate samples were determined to be lipopolysaccharide-free by *Limulus* amebocyte lysate assay (BioWhittaker, Walkersville, MD).

Vaccines. Single-stranded phosphorothioate ISS–ODN, sequence 5′-TGACTGTGAACGTTCGAGATGA-3′, were purchased from Tri-Link Biotechnology. Plasmid pACB–OVA was as described[37,38].

Peptides. $H\text{-}2^b$ MHC class I-restricted peptides were purchased from Peptido Genics Research (Fullerton, CA). OVA peptide consisted of NH_2-SIINFEKL-COOH. Influenza virus nucleoprotein (NP) peptide (negative control) consisted of NH_2-ASNENMETM-COOH.

Cytotoxic lymphocyte assay. The CTL assay was adapted from Horner and colleagues[12]. Briefly, 2×10^6 effector splenocytes were restimulated in culture for five days with 1.8×10^7 OVA peptide-pulsed stimulator splenocytes and 50 U ml^{-1} recombinant human IL-2 (BD PharMingen, San Diego, CA) in RPMI (Irvine Scientific, Santa Ana, CA) supplemented with 10% heat-inactivated FCS, 50 mM β-mercaptoethanol (Sigma), 2 mM L-glutamine, 100 U ml^{-1} penicillin, and 100 µg ml^{-1} streptomycin (RP10). After restimulation, viable lymphocytes were recovered by centrifugation over Ficoll lympholyte M (Cedarlane Laboratories, Ltd., Hornby, Ontario, Canada) at room temperature for 20 min. Cells were washed once in RP2 (RPMI + 2% FCS) and then serially diluted to several effector-to-target cell (E:T) ratios in 96-well U-bottom culture plates (Costar, Cambridge, MA) in colorless RPMI (Irvine Scientific) supplemented with 2% bovine serum albumin (BSA; Sigma), 2 mM L-glutamine, 100 U ml^{-1} penicillin, and 100 µg ml^{-1} streptomycin. Target EL4 cells were pulsed with OVA or NP peptide at 37°C for 1 h, then washed three times with colorless RPMI. Plates were incubated for 4 h, and supernatants recovered. Specific lysis was assayed with the CytoTox 96 kit (Promega, Madison, WI) according to the manufacturer's instructions.

Cytokine ELISA. Purified rat anti-mouse IFNγ capture antibody and purified, biotinylated rat anti-mouse IFNγ-detecting antibody were purchased from PharMingen. IL-4 capture and detecting antibodies (Duoset) were purchased from Genzyme (Cambridge, MA). Briefly, splenocytes were isolated as described in the previous section, and 5×10^5 splenocytes were aliquoted in triplicate into 96-well culture plates (Costar) in a total volume of 200 µl RP10 with and without 50 µg ml^{-1} ovalbumin (Sigma). Cultures were incubated at 37°C with 5% CO_2 for three days, and then aliquots of tissue culture supernatant were removed for cytokine ELISA. Half-area 96-well plates (Costar) were coated with capture antibody diluted 1:1,000 in carbonate buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$, pH 9.6), overnight at 4°C. Plates were washed with 1× BBS (160 mM NaCl, 40 mM NaOH, 200 mM boric acid, pH 8.0) and then blocked for 2 h at 37°C with blocking buffer (1% BSA in BBS). Plates were washed and incubated with tissue culture supernates diluted 1:2 in blocking buffer overnight at 4°C. Plates were washed and incubated with detecting antibody diluted 1:1,000 in blocking buffer at room temperature for 1 h. Plates were washed and incubated with streptavidin–HRP conjugate (Zymed, S. San Francisco, CA), diluted 1:2,000 in blocking buffer at room temperature for 1 h. Plates were washed and incubated with TMB substrate (Moss, Inc., Hanover, MD). The reaction was stopped with 1 M phosphoric acid (Sigma), and the plates were read at 450 nm on a Molecular Devices ThermoMax microplate reader (Sunnyvale, CA).

Ig ELISA. Alkaline phosphatase-conjugated goat anti-mouse IgG_1 and IgG_{2a} were purchased from Southern Biotechnology Associates (Birmingham, AL). Plates were coated with serum serially diluted in blocking buffer overnight at 4°C. Plates were washed and incubated with detecting antibody diluted 1:2,000 in blocking buffer. Plates were washed and incubated with 4-nitrophenyl phosphate substrate (Roche Diagnostics, Basel, Switzerland). Plates were read at 405 nm as described above.

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Acknowledgments

The authors wish to thank Tomoko Hayashi, Yucan Chiu, and Nadya Cinman for expert technical assistance, and Nancy Noon and Jane Uhle for editorial assistance. We also thank Wei Ping Feung-Leung and Tak Mak for the generous gift of CD8$^{-/-}$ mice, and Angel Porgador and Eli Gilboa for the generous gift of the E.G7–OVA cell line. This work was supported in part by grants from the National Institutes of Health (AI 40682, AI 47078, and AR 44850) and by Dynavax Technologies. H.J.C. is supported by a research resident fellowship from the Sam and Rose Stein Institute for Research on Aging (La Jolla, CA).

1. Carson, D. & Raz, E. Oligonucleotide adjuvants for T helper 1 Th1 -specific vaccination. *J. Exp. Med.* **186**, 1621–1622. (1997).
2. Krieg, A.M., Yi, A.K., Schorr, J. & Davis, H.L. The role of CpG dinucleotides in DNA vaccines. *Trends Microbiol.* **6**, 23–27 (1998).
3. Shimada, S. et al. Antitumor activity of the deoxyribonucleic acid fraction of *Mycobacterium bovis* BCG. II. Effects on various syngeneic mouse tumors. *J. Natl. Cancer Inst.* **74**, 681–688 (1985).
4. Tokunaga, T. et al. Antitumor activity of the deoxyribonucleic acid fraction from *Mycobacterium bovis* BCG. I. Isolation, physicochemical characterization, and antitumor activity. *J. Natl. Cancer Inst.* **72**, 955–962 (1984).
5. Yamamoto, S. et al. Unique palindromic sequences in synthetic oligonucleotides are required to induce INF and augment INF-mediated natural killer activity. *J. Immunol.* **148**, 4072–4076 (1992).
6. Martin-Orozco, E. et al. Enhancement of antigen-presenting cell surface molecules involved in cognate interactions by immunostimulatory DNA sequences. *Int. Immunol.* **11**, 1111–1118 (1999).
7. Krieg, A.M. et al. CpG motifs in bacterial DNA trigger direct B-cell activation. *Nature* **374**, 546–549 (1995).
8. Lipford, G.B. et al. CpG-containing synthetic oligonucleotides promote B and cytotoxic T cell responses to protein antigen: a new class of vaccine adjuvants. *Eur. J. Immunol.* **27**, 2340–2344 (1997).
9. Sato, Y. et al. Immunostimulatory DNA sequences necessary for effective intradermal gene immunization. *Science* **273**, 352–354 (1996).
10. Corr, M., Lee, D.J., Carson, D.A. & Tighe, H. Gene vaccination with naked plasmid DNA: mechanism of CTL priming. *J. Exp. Med.* **184**, 1555–1560. (1996).
11. Roman, M. et al. Immunostimulatory DNA sequences function as T helper-1-promoting adjuvants. *Nat. Med.* **3**, 849–854 (1997).
12. Horner, A.A. et al. Immunostimulatory DNA is a potent mucosal adjuvant. *Cell. Immunol.* **190**, 77–82 (1998).
13. Chu, R.S., Targoni, O.S., Krieg, A.M., Lehmann, P.V. & Harding, C.V. CpG oligodeoxynucleotides act as adjuvants that switch on T helper 1 Th1 immunity. *J. Exp. Med.* **186**, 1623–1631 (1997).
14. Davis, H.L. et al. CpG DNA is a potent enhancer of specific immunity in mice immunized with recombinant hepatitis B surface antigen. *J. Immunol.* **160**, 870–876 (1998).
15. Keene, J.-A. & Foreman, J. Helper activity is required for the in vivo generation of cytotoxic lymphocytes. *J. Exp. Med.* **155**, 768–782 (1982).
16. Mitchison, N.A. & O' Malley, C. Three-cell-type cluster of T cells with antigen-presenting cells best explain the epitope linkage and noncognate requirements of the in vivo cytolytic response. *Eur. J. Immunol.* **17**, 1579–1583 (1987).
17. Bennett, S.R.M. et al. Help for cytotoxic-T-cell responses is mediated by CD40 signaling. *Nature* **393**, 478–480 (1998).
18. Ridge, J.P., Di Rosa, F. & Matzinger, P. A conditioned dendritic cell can be a bridge between CD4$^+$ T-helper and a T-killer cell. *Nature* **393**, 474–478 (1998).
19. Schoenberger, S.P., Toes, R.E.M., van der Voort, E.I.H., Offringa, R. & Melief, C.J.M. T-cell help for cytotoxic lymphocytes is mediated by CD40–CD40L interactions. *Nature* **393**, 480–483 (1998).
20. Lin, M.S. & Chen, Y.W. B cell differentiation. II. Isotype potential of a single B cell. *Cell. Immunol.* **150**, 343–352 (1993).
21. Porgador, A., Snyder, D. & Gilboa, E. Induction of antitumor immunity using bone marrow-generated dendritic cells. *J. Immunol.* **156**, 2918–2926 (1996).
22. Lanzavecchia, A. License to kill. *Nature* **393**, 413–414 (1998).
23. Harding, F.A. & Allison, J.P. CD28–B7 interactions allow the induction of CD8$^+$ cytotoxic T lymphocytes in the absence of exogenous help. *J. Exp. Med.* **177**, 1791–1796 (1993).
24. Grewal, I.S. & Flavell, R.A. The role of CD40 ligand in costimulation and T-cell activation. *Immunol. Rev.* **153**, 85–106 (1996).
25. Sigal, L.J., Reiser, H. & Rock, K.L. The role of B7-1 and B7-2 costimulation for the generation of CTL responses in vivo. *J. Immunol.* **161**, 2740–2745 (1998).
26. Trinchieri, G. Interleukin-12: a proinflammatory cytokine with immunoregulatory functions that bridge innate resistance and antigen-specific adaptive immunity. *Ann. Rev. Immunol.* **13**, 251–276 (1995).
27. Jakob, T., Walker, P.S., Krieg, A.M., Udey, M.C. & Vogel, J.C. Activation of cutaneous dendritic cells by CpG-containing oligodeoxynucleotides: a role for dendritic cells in the augmentation of Th1 responses by immunostimulatory DNA. *J. Immunol.* **161**, 3042–3046 (1998).
28. Kovacsovics-Bankowski, M. & Rock, K.L. A phagosome-to-cytosol pathway for exogenous antigens presented on MHC class I molecules. *Science* **267**, 243–246 (1995).
29. Shen, Z., Reznikoff, G., Dranoff, G. & Rock, K. Cloned dendritic cells can present exogenous antigens on both MHC class I and class II molecules. *J. Immunol.* **158**, 2723–2730 (1997).
30. Reis e Souza, C. & Germain, R.N. Major histocompatibility complex class I presentation of peptides derived from soluble exogenous antigen by a subset of cells engaged in phagocytosis. *J. Exp. Med.* **182**, 841–851 (1995).
31. Pfeifer, J.D. et al. Phagocytic processing of bacterial antigens for class I MHC presentation to T cells. *Nature* 359–362 (1993).
32. Gromme, M. et al. Recycling MHC class I molecules and endosomal peptide loading. *Proc. Natl. Acad. Sci. USA* **96**, 10326–10331 (1999).
33. Reiss, M. Transforming growth factor-beta and cancer: a love–hate relationship? *Oncol. Res.* **9**, 447–457 (1997).
34. Botti, C., Seregni, E., Ferrari, L., Martinetti, A. & Bombardieri, E. Immunosuppressive factors: role in cancer development and progression. *Int. J. Biol. Markers* **13**, 51–69 (1998).
35. Korkolopoulous, P., Kaklamanis, L., Pezzella, F., Harris, A.L. & Gatter, K.C. Loss of antigen-presenting molecules (MHC class I and TAP-1) in lung cancer. *Brit. J. Cancer* **73**, 148–153 (1996).
36. Seliger, B. et al. Suppression of MHC class I antigens in oncogenic transformants: association with decreased recognition by cytotoxic T lymphocytes. *Exp. Hematol.* **24**, 1275–1279 (1996).
37. Dejardin, E. et al. Regulation of major histocompatibility complex class I expression by NF-kappaB-related proteins in breast cancer cells. *Oncogene* **16**, 3299–3307 (1998).

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